Kinetic and Isotope Effect Studies of Maize Phosphoenolpyruvate Carboxylase†

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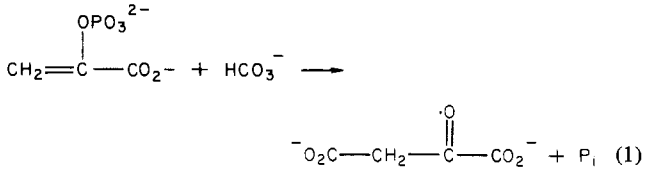
ABSTRACT: Carbon isotope effects for the carbon atom arising from bicarbonate have been measured for the phosphoenolpyruvate carboxylase from maize. At pH 7.5, 25 °C, the isotope effect is $k^{12}/k^{13} = 1.0029 \pm 0.0005$ in the presence of Mg^{2+}. The isotope effect decreases with increasing pH, reaching a value of 0.9973 at pH 10.0. All these isotope effects are relative to HCO_3^- taken as the starting state. If CO_2 is considered the starting state, the isotope effects are all inverse. These values suggest that the carboxylation of phosphoenolpyruvate occurs by way of a stepwise mechanism involving an enzyme-bound carboxyphosphate intermediate, with formation of the intermediate being the primary rate-determining step. Steady-state kinetics reveal that V_{max} is independent of pH over the range pH 7.5–10.0. V_{max}/K_m (phosphoenolpyruvate) is bell shaped in the same interval. Two pK_a values near 7 are observed; the first is attributed to ionization of the phosphate group of phosphoenolpyruvate and the second to an unidentified group on the enzyme. Activity of the enzyme also depends on protonation of a group on the enzyme with a pK_a near 10. Several metal ions were tested as activators of phosphoenolpyruvate carboxylase. Under saturating conditions, Mg^{2+} and Mn^{2+} show equal activity but different carbon isotope effects. Co^{2+} has about half the activity of Mg^{2+} and shows an inverse carbon isotope effect.

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) catalyzes the reaction of PEP[1] with HCO_3^- to form oxalacetate (eq 1)

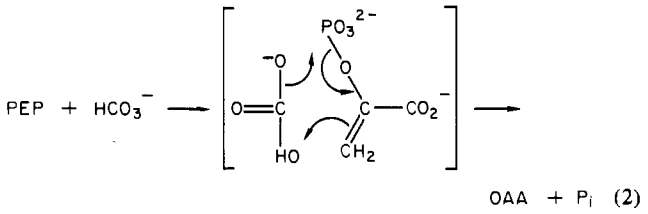


(1)

(Utter & Kolenbrander, 1972; O'Leary, 1982). The enzyme occurs in all plants (Coombs, 1979; O'Leary, 1982). It is the key carboxylating enzyme in plants which use the Hatch–Slack (C_4) pathway of photosynthetic carbon fixation and in plants which utilize Crassulacean acid metabolism (Osmond, 1978; Kluge & Ting, 1978). The enzyme also occurs in *Escherichia coli* and several other microorganisms, but the properties of that enzyme are different in a number of respects from those of the plant enzyme (Utter & Kolenbrander, 1972).

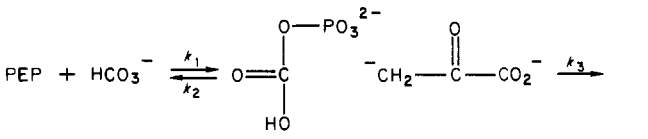
PEP carboxylase requires a divalent metal ion, but it contains no other known cofactor. The enzyme is the only non-biotin-containing carboxylase which requires HCO_3^-, rather than CO_2, as substrate (Mukerji, 1977; Cooper & Wood, 1971; Reibach & Benedict, 1977; Coombs et al., 1975). Addition of HCO_3^- is to the *si* face of PEP (Rose et al., 1969).

The carbon atom of HCO_3^- is ordinarily quite unreactive toward nucleophilic attack, and the means by which carbon–carbon bond formation is achieved by PEP carboxylase is somewhat enigmatic. The most important evidence concerning how this might be achieved is the report of Maruyama et al. (1966) that ^{18}O is transferred from $HC^{18}O_3^-$ to P_i during the carboxylation. On the basis of this observation, Maruyama et al. (1966) suggested that the carboxylation occurs by a concerted cyclic mechanism involving a single transition state (eq 2).



(2)

Although this mechanism explains the oxygen labeling results, it suffers somewhat from a lack of chemical reasonableness. A more logical alternative is a stepwise mechanism in which PEP first acts as a phosphate donor to bicarbonate, forming the anhydride intermediate carboxyphosphate (eq 3).



(3)

The other product of this first step is the enolate of pyruvate, which presumably remains bound to the enzyme adjacent to carboxyphosphate. In the second step of the reaction, the enolate attacks the carbon of carboxyphosphate, releasing P_i and forming OAA. Carboxyphosphate has been demonstrated to be an intermediate in other enzymatic reactions (Powers & Meister, 1976, 1978; Wimmer et al., 1979). This mechanism provides a clear means for utilizing the energy of the enolphosphate in catalyzing carbon–carbon bond formation and at the same time provides a means for activating the carbon atom of HCO_3^- toward nucleophilic attack.

Carbon isotope effects are a useful method for studying enzyme reaction mechanisms (O'Leary, 1977, 1978, 1980).

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[1] Abbreviations used: DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; MDH, malate dehydrogenase; PEP, phosphoenolpyruvate; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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Such studies are potentially capable of distinguishing between the concerted mechanism and the stepwise mechanism for carboxylation of PEP. The carbon isotope effect on the carboxylation of PEP has been measured several times by combustion methods in which the isotopic compositions of PEP and malate (obtained by reduction of the OAA produced by carboxylation) are obtained by combustion analysis. These compositions, together with that of the HCO_3^- used, can be used to calculate the isotope effect. Published isotope effects for this reaction are quite variable. Whelan et al. (1973) found $k^{12}/k^{13} = 1.0027 \pm 0.0044$ for PEP carboxylase from sorghum at pH 8.5, 24 °C. Reibach & Benedict (1977) reported $k^{12}/k^{13} = 1.0005$ and 1.0035 in two measurements on the enzyme from maize. Deleens et al. (1974) found $k^{12}/k^{13} = 1.0066$ and 1.0082 in two measurements on the enzyme from spinach and 1.0046 and 1.0013 for the enzyme from maize at pH 7.9, 20 °C. These studies suffer from the fact that measurements of isotope fractionation by combustion methods are very sensitive to the presence of impurities in starting materials and products. There is no evidence that the above investigators subjected their materials to rigorous purity tests. In addition, if the carboxylation of PEP is not carried to completion, the observed isotope fractionation reflects the fractionation associated not only with the HCO_3^- carbon but also with the other three carbons as well.

An alternative approach to the isotope effect has been taken by Schmidt et al. (1978), who measured the change in the isotopic composition of the HCO_3^- substrate over the course of the reaction in the presence of excess PEP. By this method, they measured an isotope fractionation $k^{12}/k^{13} = 1.002 \pm 0.0003$ at 25 °C, pH 8, for enzymes from both wheat and maize.

Because of our interest in isotope fractionations shown by C_4 and CAM plants (O'Leary & Osmond, 1980; O'Leary, 1981) and because of our interest in the mechanism of action of PEP carboxylase, we elected to measure the carbon isotope effect on the carboxylation of PEP by the same competitive methods used in our laboratory for studies of other enzymes (O'Leary, 1980). Those studies, together with related kinetic studies, are reported in this paper.

Materials and Methods

Malate dehydrogenase, malic enzyme, glutathione reductase, PEP, NADH, diethyldithiocarbamic acid, and polyvinylpyrrolidone were obtained from Sigma Chemical Co. Sephacryl S-200 was obtained from Pharmacia. DEAE-cellulose DE-52 was obtained from Whatman. Hydroxylapatite was obtained from Bio-Rad as Bio-Gel HTP. CO_2-free nitrogen gas was prepared by passing tank nitrogen through an Ascarite column.

Absorbance measurements, including those used for kinetic studies, were made on either a Cary 118 recording spectrophotometer or a Beckman DU with a Gilford optical density converter. In either instrument, the samples were maintained at 25.0 °C by using water-jacketed cell holders.

PEP carboxylase activity was measured by reducing the product oxalacetate to malate with MDH and monitoring the disappearance of NADH absorbance at 340 nm. Assay mixtures, in cuvettes with 1-cm path lengths, contained 1 mM PEP, 5 mM $NaHCO_3$, 0.5 mM $MgCl_2$, 0.2 mM NADH, 1 mM DTT, 100 mM Tris buffer at pH 8.0, and approximately 10 units of MDH in a 3-mL volume. The reactions were initiated by addition of PEP carboxylase. In kinetic experiments, the constants V_{max} and V_{max}/K_m were determined by computer fitting the velocity and concentration data (Cleland, 1979).

Preparation of PEP Carboxylase. The enzyme was prepared by a modification of the technique of Uedan & Sugiyama (1976). The extraction buffer contained 100 mM Tris, 0.5% ascorbate, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 10 mM pyruvate, 2.0 mM K_2HPO_4, and 20% glycerol at pH 7.5. The presence of glycerol in this buffer improves the yield of enzyme considerably. The general buffer contained 50 mM Tris at pH 7.5, 0.1 mM EDTA, and 10% glycerol.

Extraction. A total of 100 g of leaves harvested from *Zea mays* (strain W64-A) about 1 month of age was shredded and homogenized in a blender for 45 s with 60 mL of a 50% suspension of solid polyvinylpyrrolidone in water and 400 mL of extraction buffer. This mixture was filtered through cheesecloth and then centrifuged at 41000*g* for 30 min.

Ammonium Sulfate Fractionation. The supernate was brought to 40% saturation with solid ammonium sulfate and centrifuged at 8000*g* for 30 min. Next, the supernate was brought to 60% saturation with ammonium sulfate and centrifuged at 8000*g* for 20 min. The resulting pellet was dissolved in a minimal amount of general buffer and centrifuged at 14000*g* for 10 min. The supernate from this centrifugation contained crude PEP carboxylase.

DEAE-cellulose Chromatography. After being desalted on a Sephadex G-25 column equilibrated with general buffer, the PEP carboxylase was applied to a 3 × 22 cm column of DEAE-cellulose that had been equilibrated with general buffer. Once the column had been washed with 100 mL of general buffer, the PEP carboxylase was eluted with an 800-mL KCl gradient (0–0.2 M KCl in general buffer). The PEP carboxylase containing fractions were located and pooled.

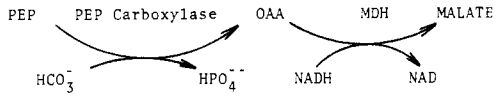
Sephacryl S-200 Chromatography. PEP carboxylase recovered from the DEAE-cellulose column was precipitated with 65% saturated ammonium sulfate, centrifuged at 14000*g* for 15 min, and redissolved in a minimal amount of general buffer. This concentrated enzyme was chromatographed on a 2.5 × 90 cm Sephacryl S-200 column that had been equilibrated with general buffer. Again the PEP carboxylase containing fractions were pooled.

Hydroxylapatite Chromatography. The PEP carboxylase was further purfied by hydroxylapatite chromatography according to the method of Mukerji (1977). PEP carboxylase isolated from the Sephacryl *S*-200 column was dialyzed against 0.01 M potassium phosphate buffer, pH 7.5, that contained 0.1 mM EDTA and 10% glycerol. The enzyme was applied to a 1.0 × 13 cm hydroxylapatite column that had been equilibrated with the phosphate buffer. After the enzyme had been washed onto the column with 22 mL of this buffer, the column was eluted with stepwise increases in the phosphate content of the buffer: 15 mL of 0.025 M, 15 mL of 0.050 M, 31 mL of 0.100 M, and 60 mL of 0.200 M phosphate. PEP carboxylase activity was eluted by both the 50 and 100 mM phosphate. No physical or kinetic differences were observed in the PEP carboxylase from these two peaks, suggesting that they were artifacts of the elution. This hydroxylapatite chromatography did not greatly increase the specific activity of the enzyme; however, it did remove traces of fumarase activity.

The PEP carboxylase used in these experiments had a specific activity of 4.1–7.0 μmol min^{-1} (mg of protein)$^{-1}$. $NaDodSO_4$–polyacrylamide gel electrophoresis of this enzyme revealed that there was one major protein contaminant, which did not interfere with the PEP carboxylase reaction. The enzyme is stable for at least a year when stored at −20 °C in general buffer to which sufficient glycerol has been added to prevent freezing.

Scheme I

1. BICARBONATE FIXATION:

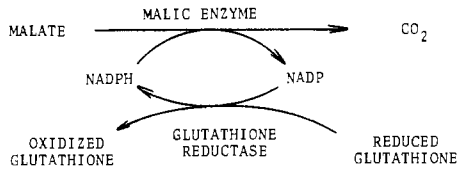


2. MALATE PURIFICATION:

   a. DOWEX-1-FORMATE CHROMATOGRAPHY.

   b. ACTIVATED CHARCOAL TREATMENT.

3. MALATE DECARBOXYLATION:



4. CO_2 RECOVERY:

   VACUUM SUBLIMATION.

5. ISOTOPIC ANALYSIS:

   ISOTOPE RATIO MASS SPECTROMETRY.

Measurement of ^{13}C Isotope Effects. The ^{13}C isotope effect on the PEP carboxylase reaction was determined by the series of procedures diagrammed in Scheme I. PEP was carboxylated by using a large excess of isotopically equilibrated CO_2/HCO_3^-. The product OAA was immediately reduced to malate in the same solution. Upon completion of these reactions, the malate was purified and decarboxylated with a malic enzyme–glutathione reductase couple (O'Leary & Osmond, 1980). Because there is an isotope effect associated with malic enzyme (Schimerlik et al., 1975; M. H. O'Leary and C. A. Roeske, unpublished results), it was necessary to assure that decarboxylation went to completion. The isotope effect was calculated by comparing the isotopic composition of the source bicarbonate with that of the CO_2 recovered from the malate.

Bicarbonate Fixation. A 100-mL sample of the reaction mixture containing 100 μmol of PEP, 5 mmol of $NaHCO_3$, 50 μmol of $MgCl_2$, 100 μmol of NADH, 20 mg of bovine serum albumin, several units of carbonic anhydrase, and 5 mmol of Hepes, pH 7.5, was equilibrated for several hours in a sealed 250-mL round-bottom flask like that described by O'Leary (1980) with the addition of a sidearm having an evacuated tube for sampling the CO_2 atmosphere. Stirring was continued until the HCO_3^- in solution and the CO_2 in the atmosphere within the flask had reached isotopic equilibrium. Two techniques were used in this equilibration, with both techniques giving similar results. In the first technique, the PEP, $MgCl_2$, Hepes, and 9 mmol of NaOH were dissolved in 100 mL of water in the round-bottom flask. A mixture of N_2 and CO_2 was bubbled through the solution until the pH stabilized. The CO_2 proportion in the gas was adjusted so that the final pH was 7.5. After the addition of NADH, serum albumin, and carbonic anhydrase, the flow of gas was stopped, the vessel was sealed, and the solution was stirred for 2 h to allow isotopic equilibration. In the second technique, Hepes, PEP, $MgCl_2$, and $NaHCO_3$ were dissolved in 100 mL of water and the pH of the solution was adjusted to 7.5 with NaOH. After the addition of NADH, serum albumin, and carbonic anhydrase, the vessel was sealed, and the solution was stirred for at least 2 h to allow equilibration.

Following equilibration by either technique, 300 units of MDH and 0.8 unit of PEP carboxylase were added by a syringe to the reaction mixture. The reaction was allowed to proceed overnight at 25 °C. Then a sample of the CO_2 above the reaction mixture was collected in the evacuated sidearm, and the reaction was stopped by the addition of 1 mL of concentrated sulfuric acid.

Malate Purification. The pH of the solution was readjusted to 7 by adding NaOH. The solution was then applied to a 1.5 × 25 cm column of Dowex-1 (formate). After the column had been washed with 100 mL of water, it was eluted with a 600-mL formic acid gradient (0–6 M). The malate-containing fractions that were eluted from the column were pooled and evaporated to dryness on a rotary evaporator. After the malate was redissolved in 100 mL of 0.1 M Hepes at pH 7.5, this solution was treated with 1 g of activated charcoal and filtered.

Malate Decarboxylation. This charcoal-treated malate solution was concentrated to a thick syrup on a rotary evaporator. This syrup was dissolved in 10 mL of 0.1 M Hepes, pH 7.5, and added to 0.2 mmol of oxidized glutathione, 0.4 μmol of NADP, and 40 μmol of $MnCl_2$ in the reaction vessel described by O'Leary (1980). This solution was sparged for 4 h with CO_2-free N_2 by using a 25-cm syringe needle. Once the needle had been removed and the vessel sealed, 5 units of malic enzyme and 38 units of glutathione reductase were added to the solution by syringe. After the decarboxylation of malate had proceeded for 48 h, the reaction was stopped by addition of 1 mL of concentrated sulfuric acid with a syringe. Enzymatic assay revealed that no malic acid remained in these solutions.

CO_2 Recovery. The sample of atmospheric CO_2 taken from the carboxylation reaction vessel and the CO_2 from the malate decarboxylation reaction were isolated according to the method described by O'Leary (1980). The sample was frozen in a dry ice–2-propanol bath, and the CO_2 was transferred under vacuum to a receiving vessel in liquid nitrogen. The sample was then thawed, and this procedure was repeated until all CO_2 had been recovered. The CO_2 was further purified by repeatedly distilling it from a vessel in dry ice–2-propanol to a receiving vessel in liquid nitrogen.

Measurement of the Isotope Effect under Different Conditions. The effect of metal ion on the isotope effect was determined by substituting $MnCl_2$ or $CoCl_2$ for $MgCl_2$ and otherwise following the procedure described above. For determination of the pH variation of the isotope effect, this procedure was modified. The Hepes buffer in the bicarbonate fixation step was replaced with Tris at pH 9.0 or glycine at pH 10.0. Furthermore, these solutions were 10% in glycerol to stabilize the PEP carboxylase.

At these high pH values, there was insufficient CO_2 in the atmosphere to allow a gaseous sample to be taken. Consequently, 2 mL of the bicarbonate fixation solution was removed with a syringe and injected into a vessel which had been flushed with CO_2-free N_2. This bicarbonate sample was then acidified with sulfuric acid, and the CO_2 was purified as described above.

Isotopic Analysis. The isotopic compositions of these CO_2 samples were determined on a Nuclide Associates RMS 6-60 isotope ratio mass spectrometer. Ratios of m/e 45 to m/e 44 were measured relative to a CO_2 standard. These ratios measure the relative abundance of $^{13}C^{16}O_2$ and $^{12}C^{17}O^{16}O$ to $^{12}C^{16}O_2$. So that the ratio of the abundance of $^{13}C^{16}O_2$ to $^{12}C^{16}O_2$ could be obtained, m/e 45/44 was corrected as described by O'Leary (1980).

Table I: Bicarbonate Carbon Isotope Effects on the Carboxylation of PEP by Maize PEP Carboxylase at pH 7.5, 25 °C[a]

isotope ratio[b] m/e 45/44 × 10^6		
CO_2 atmosphere	carbon 4 of malate	k^{12}/k^{13}
12 749	12 800	1.0037
12 744	12 806	1.0028
12 772	12 832	1.0030
12 755	12 813	1.0031
12 771	12 837	1.0027
12 771	12 839	1.0023
11 877	11 938	1.0025
11 847	11 911	1.0021
11 846	11 902	1.0029
11 845	11 906	1.0025
	av:	1.0029 ± 0.0005

[a] Reaction mixtures contained 1 mM PEP, 50 mM $NaHCO_3$, 0.5 mM $MgCl_2$, 1 mM NADH, carbonic anhydrase, malate dehydrogenase, and bovine serum albumin in 50 mM Hepes buffer, pH 7.5. [b] Mass spectrometer readings for the isotope ratio m/e 45/44 corrected to a constant value of the reference standard, but not otherwise corrected.

Table II: Bicarbonate Carbon Isotope Effects on the Carboxylation of PEP at 25 °C[a]

pH	M^{2+}	k^{12}/k^{13}
7.5	Mg^{2+}	1.0029
9.0	Mg^{2+}	1.0009
10.0	Mg^{2+}	0.9973
7.5	Mn^{2+}	1.0003
7.5	Co^{2+}	0.9981

[a] Reaction mixtures contained 1 mM PEP, 50 mM $NaHCO_3$, 0.5 mM metal, 1 mM NADH, carbonic anhydrase, malate dehydrogenase, and bovine serum albumin. Hepes buffer was used at pH 7.5, Tris at pH 9.0, and glycine at pH 10.0. At pH 9 and 10, buffers contained 10% glycerol.

For the source CO_2 samples which were obtained from the atomsphere above the bicarbonate fixation reaction mixture, the measured $^{13}CO_2$ to $^{12}CO_2$ ratio had to be corrected for the isotope effect on the equilibrium between bicarbonate in solution and CO_2 in the atmosphere by eq 4 (Mook et al., 1974).

$$\frac{H^{13}CO_3^-(aq)}{H^{12}CO_3^-(aq)} = 1.00798\frac{^{13}CO_2(atm)}{^{12}CO_2(atm)} \quad (4)$$

Once the correct ratios of $H^{13}CO_3^-$ to $H^{12}CO_3^-$ from the source and $^{13}CO_2$ to $^{12}CO_2$ from the malate had been obtained, the ^{13}C kinetic isotope effect could be calculated from

$$k^{12}/k^{13} = R_o/R_f \quad (5)$$

where R_o is the ratio of ^{13}C to ^{12}C bicarbonate of the source and R_f is the ratio of ^{13}C to ^{12}C carbon dioxide that was recovered from the malate.

Results

Carbon Isotope Effects. The bicarbonate carbon isotope effect on the carboxylation of PEP by the PEP carboxylase from *Zea mays* was calculated by comparison of the isotopic composition of the HCO_3^- source (used in large excess) with that of carbon 4 of the carboxylation product. The experimental protocol is summarized in Scheme I. Extensive measurements of the isotope effect at pH 7.5, 25 °C, were carried out by two independent investigators (J.E.R. and J. D.S.) and the results are shown in Table I. The reproducibility of these measurements is comparable to that of other measurements from this laboratory (O'Leary, 1980), and the magnitude of the isotope effect at pH 8.0 is in excellent agreement with results of Schmidt et al. (1978).

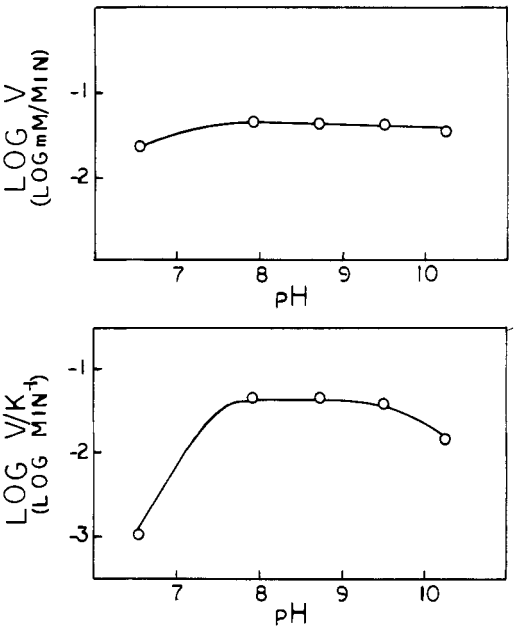


FIGURE 1: pH dependence of V_{max} and V_{max}/K_m for PEP carboxylase.

Table III: Metal Ion Dependence of PEP Carboxylase Activity[a]

metal ion	act. (μM/min)	metal ion	act. (μM/min)
Mn^{2+}	18.5	Ni^{2+}	0.94
Co^{2+}	8.66	Ca^{2+}	0.91
Mg^{2+}	4.78	Ba^{2+}	0.10
Hg^{2+}	1.22	none	0.14
Zn^{2+}	1.16		

[a] Reaction mixtures contained 0.93 mM PEP, 0.20 mM NADH, 5.0 mM $NaHCO_3$, 0.49 mM MCl_2, 100 mM Hepes buffer, pH 8.0, 11 units of malate dehydrogenase, and 0.05 unit of PEP carboxylase.

Table IV: Kinetic Constants for PEP Carboxylase at pH 8.0[a]

metal ion	$K_{m(PEP)}$ (mM)	V_{max} (μM/min)	V_{max}/K_m × 10^3 (min^{-1})
Mg^{2+}	3.3 ± 0.4	8.2 ± 0.3	2.5 ± 0.1
Mn^{2+}	0.30 ± 0.05	8.0 ± 0.4	27 ± 3
Co^{2+}	1.6 ± 0.1	4.3 ± 0.1	2.7 ± 0.2

[a] Measurements were conducted at pH 8.0, 25 °C, in 0.1 M Tris buffer containing 0.5 mM metal. All calculations are based on the concentration of free (i.e., uncomplexed) PEP.

The pH dependence and the metal ion dependence of the carbon isotope effect on the carboxylation of PEP are shown in Table II. The pH dependence of the isotope effect is similar to that seen in unpublished results of H.-L. Schmidt et al. (personal communication).

Steady-State Kinetics. The kinetics of carboxylation of PEP in the presence of saturating HCO_3^- at variable concentrations of PEP were determined as a function of pH. The derived values of V_{max} and V_{max}/K_m vs. pH are shown in Figure 1. The enzyme is quite stable under the conditions of these experiments, and no evidence for denaturation was seen at the extremes of pH.

Metal Ion Dependence. Several metal ions in addition to Mg^{2+} activate PEP carboxylase (Mukerji, 1977). For a more precise definition of the metal ion requirements of PEP carboxylase, several divalent cations were tested as activators.

The PEP carboxylase activities observed with these metals under identical conditions are compared in Table III. Mn^{2+}, Co^{2+}, and Mg^{2+} show a marked activation of the enzyme, whereas Hg^{2+}, Zn^{2+}, Ni^{2+}, and Ca^{2+} show weak activation and Ba^{2+} shows no activation.

So that a more precise comparison of the activation by Mg^{2+}, Co^{2+}, and Mn^{2+} could be obtained, the metal ion dependences of V_{max} and V_{max}/K_m were determined (Table IV). The catalytic capacity of the enzyme is equal with either Mg^{2+} or Mn^{2+} since both metals yielded similar V_{max} values. However, with Co^{2+}, the V_{max} is halved. The enzyme has similar affinities for PEP when either Co^{2+} or Mg^{2+} is the activator, but it has a 10-fold higher affinity for the substrate when Mn^{2+} is the activator as judged by the V_{max}/K_m.

Wold & Ballou (1957) have reported values for the dissociation constants of several binary metal–PEP complexes. For Mg^{2+}, Mn^{2+}, and Co^{2+}, they reported values of 5.56, 1.79, and 2.86 mM, respectively. Since these values do not parallel the variation of V_{max}/K_m with these metals, it seems unlikely that the active substrate bound by the enzyme is the binary metal–PEP complex.

Discussion

Concerted Mechanism. The concerted mechanism for carboxylation of PEP (eq 2) is characterized by a single chemical step in which all bond changes take place. In the transition state for this step, the HCO_3^- carbon is undergoing a significant change in bonding, with a new carbon–carbon bond being formed and a carbon–oxygen bond being broken. Although no precisely analogous chemical model is available which enables us to predict the magnitude of the carbon isotope effect for such a mechanism, evidence from a variety of related reactions indicates that a significant normal carbon isotope effect ($k^{12}/k^{13} > 1.0$) should be observed in the one-step mechanism. For example, carbonyl carbon isotope effects in reactions of esters with nucleophiles are large ($k^{12}/k^{13} > 1.03$) (Ropp & Raaen, 1952; O'Leary & Marlier, 1979; Marlier & O'Leary, 1981). Bimolecular substitution reactions (S_N2) at saturated carbon atoms also show large carbon isotope effects ($k^{12}/k^{13} > 1.03$) (Melander & Saunders, 1980). The carbon isotope effects observed in the carboxylation of PEP are consistently more than 10-fold smaller than these predicted values. In addition, such substitution reactions have never been observed to give rise to inverse isotope effects ($k^{12}/k^{13} < 1$), whereas the carboxylation of PEP under certain conditions shows an inverse effect. Thus, it appears that the carbon isotope effects observed for PEP carboxylase are inconsistent with the concerted mechanism (eq 2).

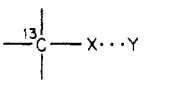
However, there is one condition under which a concerted mechanism might give rise to small carbon isotope effects. Kinetically, the carbon isotope effect on the carboxylation of PEP is an isotope effect on V_{max}/K_m; it thus reflects the isotope effects and relative rates for all steps from the binding of HCO_3^- to the enzyme through the first irreversible step in the mechanism (O'Leary, 1978). If the binding of HCO_3^- to the enzyme were extremely tight and as a result the rate of dissociation of HCO_3^- from the E·PEP·HCO_3^- complex were slow compared to the forward reaction of this complex, then a small carbon isotope effect could be observed even if the reaction were concerted and the bond-change step showed a large isotope effect. Although we do not believe that binding of HCO_3^- to the enzyme is strong enough for this possibility to obtain, available data do not allow us to exclude this possibility completely.

The pH dependences of the kinetics and isotope effects on PEP carboxylase are also inconsistent with a single rate-determining step. In a single-step mechanism, the reaction rate can change with pH without a corresponding change in isotope effect (for example, if protonation or deprotonation of enzyme or some enzyme–substrate complex produces an unreactive form), but the isotope effect cannot change with pH without a parallel change in reaction rate (the change in isotope effect indicates a change in transition state structure or concentration with pH, and this should also be reflected in the kinetics). In the case of PEP carboxylase, the carbon isotope effect shows a significant change in the pH range 7.5–10.0, whereas there is no change in V_{max} in the same interval.

Thus, it appears that the single-step concerted mechanism for carboxylation (eq 2) is inconsistent with the isotope effect data. In the following discussion, we show that the stepwise mechanism is consistent with the isotope effect data.

Stepwise Mechanism. Predicted carbon isotope effects for the stepwise mechanism (eq 3) differ according to which of the two steps is rate determining. The isotope effects predicted for the second step are about the same as those predicted for the concerted mechanism; that is, an isotope effect $k^{12}/k^{13} > 1.03$ is expected.

Prediction of isotope effects for the case in which the first step of the two-step mechanism is entirely rate determining is more difficult. The isotope effect is secondary—no bonds are made or broken to the isotopic atom—and the effect is expected to be quite small—certainly less than 1.005. However, a number of analogous isotope effects lead us to the conclusion that the isotope effect might be different from unity and in fact might be inverse. From the point of view of the isotopic atom, the transition state structure for this step shows a change in bonding between an atom attached to the isotopic atom and its neighbor (eq 6):



(6)

Several analogous reactions suggest that the change in the X–Y bond may produce a small secondary carbon isotope effect. First, the acid dissociation constant of benzoic acid shows a carboxyl carbon isotope effect $K^{12}/K^{13} = 1.001$ (Bayles et al., 1976). Second, the β-carbon isotope effect on the decarboxylation of malonic acid (eq 7)

$$^-O_2{}^{13}C—CH_2—CO_2^- \rightarrow {}^-O_2{}^{13}C—CH_3 + CO_2 \quad (7)$$

is about $k^{12}/k^{13} = 1.005$ (Melander & Saunders, 1980). Third, the reaction of acetophenone with phenylhydrazines shows a methyl carbon isotope effect $k^{12}/k^{14} = 0.992$ and an isotope effect at carbon 1 of the benzene ring of $k^{12}/k^{14} = 0.996$ (Raaen et al., 1960, 1963). Fourth, the methyl carbon isotope effect on the hydrazinolysis of methyl benzoate (the transition state is characterized by a large degree of carbonyl carbon–oxygen bond breaking) is $k^{12}/k^{13} = 1.002$ (O'Leary & Marlier, 1979).

The conclusion which emerges from all of these data is that a significant carbon isotope effect can be observed in reactions in which the X–Y bond is made or broken (eq 6); when the bond is being broken, the isotope effect is normal, whereas when the bond is being made, the isotope effect is inverse. The maximum isotope effect observable in such a case may be near $k^{12}/k^{13} = 1.005$ in a bond-breaking reaction or 0.995 in a bond-making reaction, although these values must be considered tentative. Thus, in the case of PEP carboxylase, we predict that if the first step of the two-step mechanism is rate determining, a small inverse isotope effect should be observed. If the second step is rate determining, a substantial normal

isotope effect should be observed.

If neither step in the two-step mechanism is entirely rate determining, then the observed isotope effect will reflect the isotope effects and relative rates of the two steps (eq 8).

$$k^{12}/k^{13} = (k_1^{12}/k_1^{13})\frac{[(k_3^{12}/k_3^{13})/(k_2^{12}/k_2^{13})] + (k_3^{12}/k_2^{12})}{1 + (k_3^{12}/k_2^{12})} \quad (8)$$

We assume in the derivation of eq 8 that dissociation of HCO_3^- from the enzyme·PEP·HCO_3^- complex is rapid compared to k_1.

Within the framework of eq 8 and the predictions regarding the separate isotope effects made previously, it is clear that at pH 7.5 in the presence of Mg^{2+} the second step in the reaction is not rate determining. However, if the first step were completely rate determining, we might expect to see a small inverse isotope effect. The small normal effect observed probably arises from the fact that the first step is mostly rate limiting, but not entirely so. A value of k_3/k_2 near 10 would explain the results.

The rate of carboxylation of PEP is essentially independent of pH in the range pH 7.5–10.0 whereas the carbon isotope effect changes significantly in this interval, becoming inverse at pH 10. This combination of effects is most easily explained as a change in relative rates of the two steps without a net change in rate-determining step. If, as previously suggested, the first step is rate determining at pH 7.5 and $k_3/k_2 \approx 10$, then as the pH increases, the first step continues to be rate determining, and the rate of this step is independent of pH. However, as the pH increases, the rate of the second step increases, and thus, k_3/k_2 increases. As a result, the observed isotope effect approaches the isotope effect on the first step, but no change in V_{max} is observed. The inverse carbon isotope effect observed at pH 10 is consistent with such a mechanism.

The smaller isotope effects observed with Mn^{2+} and Co^{2+} presumably reflect a change in relative rates of the two steps. The fact that V_{max} for Mn^{2+} is the same as that for Mg^{2+} whereas the isotope effect is significantly smaller indicates that this metal substitution has little effect on the rate of the first step and a larger effect on the rate of the second. Substitution of Co^{2+} appears to decrease the rate of the first step slightly but to significantly increase the rate of the second step.

pH Dependence. Numerous measurements indicate that PEP carboxylase has a pH optimum in the range pH 8–9 (Coombs et al., 1973; Mukerji & Yang, 1974; Huber & Edwards, 1975; Bandurski, 1955; Maruyama & Lane, 1962; Maruyama et al., 1966; Smith et al., 1979), but with one exception (Smith et al., 1979), no account has been taken of separate pH effects on V_{max} and V_{max}/K_m.

V_{max} shows little variation with pH in the range tested in this experiment. In contrast, V_{max}/K_m shows a decrease at pHs both below 7.5 and above 9.5. V_{max}/K_m decreases much more rapidly at low pHs than one would expect from the protonation of a single base. Since this parameter reflects the affinity of PEP for the enzyme, its pH dependence implies that a group with a pK around 10 must be protonated and probably two groups with pKs around 7.5 must be unprotonated for the enzyme to bind PEP (Cleland, 1977). Normally the pK_a of bicarbonate would appear in the pH dependence of V_{max}/K_m around pH 6.37. However, in this experiment, a large excess of bicarbonate relative to its K_m was used so that the apparent pK of bicarbonate would be shifted to a much lower pH. On the other hand, the phosphate pK of PEP should appear in this profile somewhere around pH 6.35. Probably this phosphate group is responsible for one of the ionizations seen around pH 7. This suggests that the form of PEP bound by the enzyme has a fully deprotonated phosphate group. The other group that must be unprotonated and the group of pK_a $\approx$ 10 that must be protonated for PEP binding must be associated with the enzyme or the metal ion.

Several intriguing questions surround the protonation state of the proposed intermediate. In the first place, although the enolate of pyruvate is highly basic, it appears that it is not protonated in the active site to any appreciable extent. Further, the carboxyphosphate intermediate (eq 3) is formed with a neutral carboxyl group and a doubly charged phosphate. This is not the thermodynamically favored form, though it is probably the correct form for reaction with the enolate. Either the enzyme must control these protonations (perhaps by formation of a metal–enolate complex) or else the lifetime of the intermediate is too short for proton transfer to occur.

Conclusions

The small carbon isotope effects observed in the carboxylation of PEP are inconsistent with the proposed concerted mechanism (Maruyama et al., 1966) for formation of OAA. Instead, it appears that the carboxylation occurs in two steps with a carboxyphosphate intermediate. This conclusion is strengthened by the difference in pH dependence between the steady-state kinetic parameters and the carbon isotope effects.

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Application of High-Field Proton Magnetic Resonance Spectroscopy in the Structural Determination of Membrane-Derived Sindbis Virus Glycopeptides†

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ABSTRACT: Sindbis virus membrane glycopeptides have been purified in chemical quantities and their oligosaccharide structures analyzed by ^{1}H NMR spectroscopy at 360 MHz. Interpretable spectra could be obtained with approximately 100 μg of oligosaccharide. Spectral analysis of the sialyl glycopeptides S1, S2, and S3 at high and low temperatures confirms their structures to be NANAα(2,3)Galβ(1,4)-GlcNAcβ(1,2)Manα(1,6)-[NANAα(2,3)Galβ(1,4)-GlcNAcβ(1,2)Manα(1,3)]-Manβ(1,4)GlcNAcβ(1,4)-[Fucα-(1,6)]-GlcNAcβ1-Asn. These are heterogeneous with respect to sialic acid (NANA). Spectra of two *endo*-β-*N*-acetylglucosaminidase products of the S4 glycopeptides are reported. The interpretation of these spectra is consistent with $Man_5GlcNAc$ and $Man_7GlcNAc$ oligosaccharide structures. Their chemical shifts are essentially identical with those reported for ovalbumin glycopeptides of the same composition, with exception to the perturbations arising from their oligosaccharide nature.

Interest in the elucidation of oligosaccharide structures in membrane glycoproteins derives from accumulating evidence that the oligosaccharides of membrane glycoproteins may be involved in cell surface recognition events [reviewed by Frazier & Glaser (1979); Neufeld & Ashwell, 1980]. In addition, some studies suggest that glycosylation is required for the establishment and preservation of specific protein conformation (Gibson et al., 1981), which in turn affects the biological properties of some glycoproteins (Kaluza et al., 1980). In the latter studies, a quite subtle alteration in glycosylation changed antigenic characteristics of Semliki Forest virus intracellular proteins p62 and E1. Presumably such alterations might include changes in the ratio of higher mannose polymers to lower mannose polymers previously described in the four major Sindbis virus (SbV) S4 glycopeptides of glycoproteins E1 and E2 (Hakimi & Atkinson, 1980a).

To better detect fine structural details, we have used high-field ^{1}H NMR[1] analysis of membrane glycopeptides. Such spectroscopy at 360 MHz is a powerful tool in structural determination of oligosaccharides and glycopeptides [for review, see Carver & Grey (1981); Montreuil, 1980]. Unlike classical methods of oligosaccharide structural analysis, ^{1}H NMR spectroscopy is a nondestructive technique which yields information on sugar composition, anomeric configuration, linkage, number of antennae, some sequential details, and tertiary structure of oligosaccharides. It also can be used to assay sample homogeneity during purification (Cohen & Ballou, 1980; Atkinson et al., 1981) which is again illustrated in this report, because mixtures give rise to spectra with resonances at nonintegral ratios of intensity. Considerable ^{1}H NMR spectra of sialyl-type glycopeptides have been published (Dorland et al., 1978; Fournet et al., 1978; Narasimhan et al., 1980; Vliegenthart et al., 1981), and chemical shift assignments have been established. Such detailed analysis on the oligomannosidic-type glycopeptides has also been reported (Gorin et al., 1969; Cohen & Ballou, 1980; Carver et al., 1981;

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[1] Abbreviations used: ^{1}H NMR, proton magnetic resonance; Man, mannose; Gal, galactose; Fuc, fucose; GlcNAc, *N*-acetylglucosamine.

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